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(57) Abstract

The invention relates to nucleic acid sequences isolated from Ashbya gossypii that encode proteins essential for fungal growth. The invention also includes the methods of using these proteins pesticide targets, particularly fungicide targets, based on the essentiality of the gene for normal growth and development. The invention is also useful as a screening assay to identify inhibitors that are potential pesticides, particularly fungicides.

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FUNGAL GENES REQUIRED FOR NORMAL GROWTH AND DEVELOPMENT

The invention relates to nucleic acid sequences isolated from Ashbya gossypii that encode proteins essential for fungal growth. The invention also includes the methods of using these proteins pesticide targets, particularly fungicide targets, based on the essentiality of the gene for normal growth and development. The invention is also useful as a screening assay to identify inhibitors that are potential pesticides, particularly fungicides.

The phytopathogenic fungus Ashbya gossypii is a filamentously growing ascomycete that was first isolated as a plant pathogen in tropical and sub-tropical regions. It infects the seed capsule of cotton plants and has also been isolated from tomatoes and citrus fruits. The infection of the seed capsule is caused by transmission of A. gossypii mycelium pieces or spores by stinging-sucking insects and causes a disease called stigmatomycosis. Presently, A. gossypii represents the most compact eukaryotic genome, compared to genome sizes of 12.5 Mb for S. cerevisiae (Chu et al., 1986), 31.0 Mb for Aspergillus nidulans (Brody and Carbon, 1989) and 47.0 Mb for Neurospora crassa (Orbach et al., 1988).

A. gossypii is systematically grouped to the endomycetales belonging to the family of spermophthoraceae. This classification is based on the observation that the spores that develop in hyphal compartments called sporangia look like ascospores, which are defined as endproducts of meiosis.

Since Ashbya gossypii is a filamentous ascomycete, and is capable of growing only by filamentous (hyphal) growth, fungal targets found in this model organism are predictive of targets which will be found in other pathogens, the vast majority of which grow in a filamentous fashion. .

DEFINITIONS

For clarity, certain terms used in the specification are defined and presented as follows:

Chimeric: is used to indicate that a DNA sequence, such as a vector or a gene, is comprised of more than one DNA sequences of distinct origin which are fused together by recombinant DNA techniques resulting in a DNA sequence, which does not occur naturally, and which particularly does not occur in the plant to be transformed.

Co-factor: natural reactant, such as an organic molecule or a metal ion, required in an enzyme-catalyzed reaction. A co-factor is e.g. NAD(P), riboflavin (including FAD and FMN), folate, molybdopterin, thiamin, biotin, lipoic acid, pantothenic acid and coenzyme A, S-adenosylmethionine, pyridoxal phosphate, ubiquinone, menaquinone. Optionally, a co-factor can be regenerated and reused.

Enzyme activity: means herein the ability of an enzyme to catalyze the conversion of a substrate into a product. A substrate for the enzyme comprises the natural substrate of the enzyme but also comprises analogues of the natural substrate which can also be converted by the enzyme into a product or into an analogue of a product. The activity of the enzyme is measured for example by determining the amount of product in the reaction after a certain period of time, or by determining the amount of substrate remaining in the reaction mixture after a certain period of time. The activity of the enzyme is also measured by determining the amount of an unused co-factor of the reaction remaining in the reaction mixture after a certain period of time or by determining the amount of used co-factor in the reaction mixture after a certain period of time. The activity of the enzyme is also measured by determining the amount of a donor of free energy or energy-rich molecule (e.g. ATP, phosphoenolpyruvate, acetyl phosphate or phosphocreatine) remaining in the reaction mixture after a certain period of time or by determining the amount of a used donor of free energy or energy-rich molecule (e.g. ADP, pyruvate, acetate or creatine) in the reaction mixture after a certain period of time.

Expression: refers to the transcription and/or translation of an endogenous gene or a transgene in plants. In the case of antisense constructs, for example, expression may refer to the transcription of the antisense DNA only.

Gene: refers to a coding sequence and associated regulatory sequences wherein the coding sequence is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense RNA or antisense RNA. Examples of regulatory sequences are promoter sequences, 5' and 3' untranslated sequences and termination sequences. Further elements that may be present are, for example, introns.

Heterologous DNA Sequence: a DNA sequence not naturally associated with a host cell into which it is introduced, including non-naturally occurring multiple copies of a naturally occurring DNA sequence.

Homologous DNA Sequence: a DNA sequence naturally associated with a host cell into which it is introduced.

Isogenic: plants which are genetically identical, except that they may differ by the presence or absence of a transgene.

Isolated: in the context of the present invention, an isolated DNA molecule or an isolated enzyme is a DNA molecule or enzyme that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated DNA molecule or enzyme may exist in a purified form or may exist in a non-native environment such as, for example, a transgenic host cell.

Mature protein: protein which is normally targeted to a cellular organelle, such as a chloroplast, and from which the transit peptide has been removed.

Minimal Promoter: promoter elements, particularly a TATA element, that are inactive or that have greatly reduced promoter activity in the absence of upstream activation. In the presence of a suitable transcription factor, the minimal promoter functions to permit transcription.

Modified Enzyme Activity: enzyme activity different from that which naturally occurs in a plant (i.e. enzyme activity that occurs naturally in the absence of direct or indirect manipulation of such activity by man), which is tolerant to inhibitors that inhibit the naturally occurring enzyme activity.

Recombinant DNA molecule: a combination of DNA sequences that are joined together using recombinant DNA technology

Recombinant DNA technology: procedures used to join together DNA sequences as described, for example, in Sambrook et al.,1989, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

Significant Increase: an increase in enzymatic activity that is larger than the margin of error inherent in the measurement technique, preferably an increase by about 2-fold or greater of the activity of the wild-type enzyme in the presence of the inhibitor, more preferably an increase by about 5-fold or greater, and most preferably an increase by about 10-fold or greater.

Significantly less: means that the amount of a product of an enzymatic reaction is larger than the margin of error inherent in the measurement technique, preferably a decrease by about 2-fold or greater of the activity of the wild-type enzyme in the absence of the inhibitor, more preferably an decrease by about 5-fold or greater, and most preferably an decrease by about 10-fold or greater.

In its broadest sense, the term "substantially similar", when used herein with respect to a nucleotide sequence, means a nucleotide sequence corresponding to a reference

nucleotide sequence, wherein the corresponding sequence encodes a polypeptide having substantially the same structure and function as the polypeptide encoded by the reference nucleotide sequence, e.g. where only changes in amino acids not affecting the polypeptide function occur. Desirably the substantially similar nucleotide sequence encodes the polypeptide encoded by the reference nucleotide sequence. The term "substantially similar" is specifically intended to include nucleotide sequences wherein the sequence has been modified to optimize expression in particular cells. The percentage of identity between the substantially similar nucleotide sequence and the reference nucleotide sequence desirably is at least 65%, more desirably at least 75%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, yet still more preferably at least 99%. Sequence comparisons are carried out using a Smith-Waterman sequence alignment algorithm (see e.g. Waterman, M.S. Introduction to Computational Biology: Maps, sequences and genomes. Chapman & Hall. London: 1995. ISBN 0-412-99391-0, or at _ HYPERLINK "http://www-hto.usc.edu/software/seqaln/index.html" __http://www-hto.usc.edu/software/seqaln/index.html_). The localS program, version 1.16, is used with following parameters: match: 1, mismatch penalty: 0.33, open-gap penalty: 2, extended-gap penalty: 2. A nucleotide sequence "substantially similar" to reference nucleotide sequence hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C.

The term "substantially similar", when used herein with respect to a protein, means a protein corresponding to a reference protein, wherein the protein has substantially the same structure and function as the reference protein, e.g. where only changes in amino acids sequence not affecting the polypeptide function occur. When used for a protein or an amino acid sequence the percentage of identity between the substantially similar and the reference protein or amino acid sequence desirably is at least 52%, more desirably 65%, more desirably at least 75%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, yet still more preferably at least 99%.

Substrate: a substrate is the molecule that the enzyme naturally recognizes and converts to a product in the biochemical pathway in which the enzyme naturally carries out its function, or is a modified version of the molecule, which is also recognized by the enzyme and is converted by the enzyme to a product in an enzymatic reaction similar to the naturally-occurring reaction.

Tolerance: the ability to continue normal growth or function when exposed to an inhibitor or herbicide in an amount sufficient to suppress the normal growth or function of native, unmodified plants.

Transformation: a process for introducing heterologous DNA into a cell, tissue, or plant. Transformed cells, tissues, or plants are understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof.

Transgenic: stably transformed with a recombinant DNA molecule that preferably comprises a suitable promoter operatively linked to a DNA sequence of interest.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO:1comprises a AG001 coding region

SEQ ID NO:2comprises an amino acid sequence encoded by the coding region of SEQ ID NO:1

SEQ ID NO:3 comprises a AG002 coding region.

SEQ ID NO:4 comprises an amino acid sequence encoded by the coding region of SEQ ID NO:3.

SEQ ID NO:5 comprises a AG003 coding region.

SEQ ID NO:6 comprises an amino acid sequence encoded by the coding region of SEQ ID NO:5.

SEQ ID NO:7 comprises a AG004 coding region.

SEQ ID NO:8 comprises an amino acid sequence encoded by the coding region of SEQ ID NO:7.

SEQ ID NO:9 comprises a AG005 coding region.

SEQ ID NO:10 comprises an amino acid sequence encoded by coding region of SEQ ID NO:9.

SEQ ID NO:11 comprises a AG006 coding region.

SEQ ID NO:12 comprises an amino acid sequence encoded by coding region of SEQ ID NO:11.

It is an object of the invention to provide an effective and beneficial method to identify novel pesticides, particularly fungicides. A feature of the invention is the identification of genes having a putative activity based on their homology to yeast genes. Genes of the invention comprise a putative GTP binding protein genes (herein referred to as AG001 and AG002 genes), putative GTPase activating protein genes(AG003 and AG004), putative phosphatidylinositol-4 kinase protein gene (AG005) and putative cytokinesis gene (AG006). Another feature of the invention is the discovery that the genes of the invention, AG001 (SEQ ID. NO: 1), AG002 (SEQ Id. NO 3):, AG003 (SEQ ID. NO: 5), AG004 (SEQ ID. NO: 7), AG005 (SEQ *Id.* NO: 9) and AG006(SEQ ID. NO: 11) are essential for fungal growth and development. An advantage of the present invention is that the newly discovered essential genes containing a novel fungicidal mode of action enables one skilled in the art to easily and rapidly identify novel fungicides.

One object of the present invention is to provide essential genes in fungi for assay development to detect inhibitory compounds with pesticidal, particularly fungicidal activity. Genetic results show that when AG001, AG002, AG003, AG004, AG005 and AG006 are mutated in Ashbya gossypii, the resulting phenotype is at best suppressed growth and at worst lethal. Suppressed growth as used herein results in a growth rate of half the growth rate observed in wild type or lower where 10% that of the wild-type growth rate was observed or no growth was macroscopically detected at all . Applicants further observed that when AG001, AG002, AG003, AG004, AG005 and AG006 are mutated in Ashbya gossypii abnormal filament development was observed. This suggests a critical role for the gene products encoded by the mutated genes.

The inventors of the present invention have demonstrated that the gene products of the invention are essential in Ashbya gossypii. This implies that chemicals which inhibit the function of the protein in fungi, particularly, filamentous fungi, are likely to have detrimental effects on fungi and are potentially good fungicide candidates. The present invention therefore provides methods of using a purified protein encoded by the gene sequence described below to identify inhibitors thereof, which can then be used as fungicides to suppress the growth of pathogenic fungi.

Pathogenic fungi is defined as those capable of colonizing a host and causing disease. Examples of fungal pathogens include plant pathogens such as Septoria tritici, Stagnospora nodorum, Botrytis cinerea, Fusarium graminearum, Magnaporthe grisea, Cochliobolus heterostrophus, Colletotrichum heterostrophus, Ustilago maydis, Erisyphe

graminis, plant pathogenic oomycetes such as Pythium ultimum and Phytophthora infestans, and human pathogens such as Candida albicans and Aspergillus fumigatus

The present invention discloses novel nucleotide sequences derived from Ashbya gossypii designated as the AG001gene, the AG002 gene, the AG003 gene, the AG004 gene, the AG005 gene and the AG006 gene. The nucleotide sequence of the genomic clones are set forth in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 and SEQ ID NO: 11 respectively. The amino acid sequence encoded by the above sequences are set forth in SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, SEQ ID NO:10 or SEQ ID NO:12 . The present invention also includes nucleotide sequences substantially similar to those set forth in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11 and amino acid sequences substantially similar to those set out in SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, SEQ ID NO:10 or SEQ ID NO:12

The present invention also encompasses fungal proteins whose amino acid sequence are substantially similar to the amino acid sequences set forth in SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, SEQ ID NO:10 or SEQ ID NO:12. Encompassed by the present invention is a nucleotide sequence having a 20 base pair nucleotide portion identical in sequence to a 20 consecutive base pair portion of a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11. Preferred is a nucleotide sequence having a base pair nucleotide portion identical in sequence to a 18 consecutive base pair portion of the sequence set forth in SEQ ID NO: 1. Preferred is a nucleotide sequence having a base pair nucleotide portion identical in to a consecutive 9 base pair portion of the sequence set forth in SEQ ID NO:3. . Preferred is a nucleotide sequence having a base pair nucleotide portion identical in sequence to a 15 consecutive base pair portion of the sequence set forth in SEQ ID NO:5. . Preferred is a nucleotide sequence having a base pair nucleotide portion identical in sequence to a 14 consecutive base pair portion of the sequence set forth in SEQ ID NO:7. . Preferred is a nucleotide sequence having a base pair nucleotide portion identical in sequence to a 12 consecutive base pair portion of the sequence set forth in SEQ ID NO:9. . Preferred is a nucleotide sequence having a base pair nucleotide portion identical in sequence to a consecutive 10 base pair portion of the sequence set forth in SEQ ID NO:11.

In a particular embodiment, the present invention encompasses nucleic acid sequences and amino acid sequences of filamentous fungi. Preferred is a nucleotide sequence wherein the fungus is Ashbya gossypii.
Further encompassed by the invention is a chimeric gene comprising a promoter operably linked to a nucleotide sequence according to the invention. Preferred is a chimeric gene wherein the promoter is an inducible promoter. A further embodiment of the invention is a recombinant vector comprising a chimeric gene according to the invention wherein said vector is capable of being stably transformed into a host cell.
Also included in the invention is a host cell comprising the vector according to the invention, wherein the nucleotide sequence is expressible in the host cell. Preferred is a host cell , wherein the host cell is eukaryotic. Preferred is a host cell, wherein the host cell is selected from the group consisting of a yeast cell and a fungal cell. More preferred is a host cell, wherein the host cell is a filamentous fungal cell. Particularly preferred is a host cell according to claim 24, wherein the host cell is an Ashbya gossipii cell.
Preferred is a host cell wherein the host cell is a prokaryotic cell. More preferred is a host cell wherein the host cell is a bacterial cell.

The present invention also includes methods of using the AG001 to AG006 gene products as fungicide targets, based on the essentiality of the genes for normal growth and development. Normal growth and development is defined as a growth rate substantially similar to that observed in wild type fungus, preferably greater than at least 50% the growth rate observed in wild type fungus and particularly greater than 10% the growth rate obeserved in wild type fungus. Normal growth and development may also be defined, when used in relation to filamentous fungi, as normal filament development (including normal septation and normal nuclear migration and distribution), normal sporulation, and normal production of any infection structures (e.g. appressoria). Conversely suppressed or inhibited growth as used herein is defined as less than half the growth rate observed in wild type or lower where 10% that of the wild-type growth rate was observed or no growth was macroscopically detected at all or abnormal filament development.

Furthermore, the invention can be used in screening assays to identify inhibitors that are potential pesticides, particularly fungicides. Encompassed by the present invention is the use of sequences selected from the attached Sequence Listing to identify substances having antifungal activity; the use of sequences selected from the attached Sequence Listing to identify substances having pesticidal, particularly fungicidal, activity.

Further comprised is the use of an a DNA sequence selected from the Sequence Listing and variants thereof in a screening method for identifying compounds capable of inducing broad spectrum disease resistance in plants.

Encompassed by the invention is a process for identifying compounds having fungicidal activity comprising the steps of:

a) combining a protein according to claim 16 and a compound to be tested for the ability to bind to the protein, under conditions having conducive binding,

b) selecting a compound identified by step a) that is capable of binding the protein;

c) applying the identified compound from step b) to a fungus to test for fungicidal activity; and

d) selecting compounds having fungicidal activity.

Encompassed by the present invention is a process for identifying an inhibitor of a protein activity having an amino acid sequence according to claim 16 comprising:

a) introducing SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 or SEQ ID NO: 11 or nucleotide sequences substantially similar thereto into a host, such that the sequence is functionally expressible;

b) combining the host cell of step a) with a compound to be tested for ability to inhibit the protein activity;

c) over expressing the nucleotide sequence of step a);

c) measuring the host cell growth in stepc); and

d) selecting the compound that inhibits or suppresses host cell's normal growth or development in step c).

Further encompassed is a compound having fungicidal activity which compound can be identified by a process for identifying compounds having fungicidal activity according to the invention.

Further encompassed is a method of suppressing growth of a fungus comprising applying to the fungus a compound that inhibits the activity of a protein comprising the amino acid sequence according to the invention in an amount sufficient to suppress the growth of the fungus.

In a further embodiment according to the invention, a DNA sequence selected from the Sequence Listing may also be used for distinguishing among different species of plant pathogenic fungi and for distinguishing fungal pathogens from other pathogens such as bacteria.

In another preferred embodiment, the present invention describes a method for identifying chemicals having the ability to inhibit any one or more of AG001, AG002, AG003, AG004, AG005 and AG006 activity in fungi preferably comprising the steps of: a) obtaining transgenic fungus and/or fungal cell, preferably stably transformed, comprising a non-native nucleotide sequence or an endogenous nucleotide sequences operably linked to non-native promoter, preferably an inducible promoter, encoding an enzyme having and activity and capable of overexpressing an enzymatically active AG001, AG002, AG003, AG004, AG005 or AG006 gene product where overexpression of the gene product is suppresses or inhibits the normal growth and development of the fungus; b) applying a compound to the transgenic fungus and/or fungal cell c) determining the growth and/or development of the transgenic fungus and/or fungal cell after application of the compound; d) comparing the growth and/or development of the transgenic fungus and/or fungal cell after application of the chemical to the growth and/or development of the corresponding transgenic fungus and/or fungal cell to which the compound was not applied; and e) selecting compound that does not results in reduction of the suppressed or inhibited growth and/or development in the transgenic fungus and/or fungal cell in comparison to the untreated transgenic fungus and/or fungal cell.

In a preferred embodiment, the proteins having AG001, AG002, AG003, AG004, AG005 or AG006 activities are encoded by nucleotide sequence derived from fungi, preferably filamentous fungi, particularly from Ashbya gossypii, desirably identical or substantially similar to the nucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO: 7, SEQ ID NO:9 or SEQ ID NO:11. In another embodiment, the proteins having AG001, AG002, AG003, AG004, AG005 or AG006 activity are encoded by nucleotide sequences capable of encoding the amino acid sequences of: SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, SEQ ID NO:10 or SEQ ID NO:12. In yet another embodiment, the proteins having AG001, AG002, AG003, AG004, AG005 or AG006 activity have amino acid sequences identical or substantially similar to the amino acid sequence set forth in SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, SEQ ID NO:10 or SEQ ID NO:12 respectively.

The invention also provides a method for suppressing the growth of a fungus comprising the step of applying to the fungus a compound that inhibits the naturally occurring AG001, AG002, AG003, AG004, AG005 and/or AG006 activity in the fungus.

Other objects and advantages of the present invention will become apparent to those skilled in the art from a study of the following description of the invention and non-limiting examples.

Essentiality of the AG001, AG002, AG003, AG004, AG005 and AG006 Genes in Ashbya gossypii Demonstrated by Gene Disruption

Owing to the provision within the scope of this invention of a novel and powerful gene disruption process, there is no longer a need to know the exact biological function of the protein product encoded by a gene comprising one of the A. gossypii DNA sequences provided herein.

As shown in the examples below, the identification of novel gene structures, as well as the essentiality of the AG001, AG002, AG003, AG004, AG005 and AG006 genes for normal fungal growth and development, have been demonstrated for the first time in Ashbya gossypii using gene disruption techniques. Having established the essentiality of AG001, AG002, AG003, AG004, AG005 and AG006 function in fungi and having identified the nucleic acid sequences encoding these essential activities, the inventors thereby provide an important and sought after tool for new pesticide, particularly fungicide, development.

Recombinant Production of and Uses Thereof

For recombinant production of AG001, AG002, AG003, AG004, AG005 and AG006 in a host organism, a nucleotide sequence encoding AG001, AG002, AG003, AG004, AG005 or AG006 protein is inserted into an expression cassette designed for the chosen host and introduced into the host where it is recombinantly produced. The choice of specific regulatory sequences such as promoter, signal sequence, 5' and 3' untranslated sequences, and enhancer appropriate for the chosen host is within the level of skill of the routineer in the art. The resultant molecule, containing the individual elements operably linked in proper reading frame, may be inserted into a vector capable of being transformed into the host cell. Suitable expression vectors and methods for recombinant production of proteins are well known for host organisms such as E. coli, yeast, and insect cells (see, e.g., Luckow and Summers, Bio/Technol. 6: 47 (1988), and baculovirus expression vectors,

e.g., those derived from the genome of Autographica californica nuclear polyhedrosis virus (AcMNPV). A preferred baculovirus/insect system is pAcHLT (Pharmingen, San Diego, CA) used to transfect Spodoptera frugiperda Sf9 cells (ATCC) in the presence of linear Autographa californica baculovirus DNA (Pharmigen, San Diego, CA). The resulting virus is used to infect HighFive Tricoplusia ni cells (Invitrogen, La Jolla, CA). Further preferred expression systems are commercially available such as Baculovirus expression systems: MaxBac 2.0 kit; Invitrogen, Calsbad, CA;BacPAK Baculovirus Expression System; CLONTECH, Palo Alto, CA; for Yeast expression vectors: pYEUra3; CLONTECH, Palo Alto, CA; EasySelect Pichia expression kit; Invitrogen, Calsbad, CA;ESP Yeast Protein Expression and Purification System; Stratagene, La Jolla, CA; E. coli expression vectors: pKK233-2; CLONTECH, Palo Alto, CA; pET3 series vectors; Stratagene, La Jolla, CA.

In a preferred embodiment, the nucleotide sequence encoding a protein having AG001, AG002, AG003, AG004, AG005 Or AG006 activity is derived from an eukaryote, such as a mammal, a fly or a yeast, but is preferably derived from a fungus, particularly a filamentous fungus. In a further preferred embodiment, the nucleotide sequence is identical or substantially similar to the nucleotide sequence set forth in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 or SEQ ID NO: 11 , or encodes a protein having AG001, AG002, AG003, AG004, AG005 or AG006 activity, whose amino acid sequence is identical or substantially similar to the amino acid sequence set forth in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10 or SEQ ID NO: 12 respectively. The nucleotide sequences set forth in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11 encode the protein comprising amino acid sequence is set forth in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10 OR SEQ ID NO: 12. In another preferred embodiment, the nucleotide sequence is derived from a prokaryote, preferably a bacteria.

Recombinantly produced AG001, AG002, AG003, AG004, AG005, or AG006 is isolated and purified using a variety of standard techniques. The actual techniques that may be used will vary depending upon the host organism used, whether the protein is designed for secretion, and other such factors familiar to the skilled artisan (see, e.g. chapter 16 of Ausubel, F. et al., "Current Protocols in Molecular Biology", pub. by John Wiley & Sons, Inc. (1994).

Assays for Characterizing the AG001, AG002, AG003, AG004, AG005 and AG006 Proteins

Recombinantly produced AG001, AG002, AG003, AG004, AG005 and AG006 proteins are useful for a variety of purposes. For example, they can be used in in vitro assays to screen known pestcidal, particularly fungicidal chemicals whose target has not been identified to determine if they inhibit AG001, AG002, AG003, AG004, AG005 or AG006. Such in vitro assays may also be used as more general screens to identify chemicals that inhibit such enzymatic activities and that are therefore novel pesticide, particularly fungicide, candidates. Alternatively, recombinantly produced AG001, AG002, AG003, AG004, AG005 or AG006 proteins may be used to elucidate the complex structure of these molecules and to further characterize their association with known inhibitors in order to rationally design new inhibitory pesticides, particularly fungicides. Nucleotide sequences substantially similar to SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11 and proteins substantially similar to SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10 OR SEQ ID NO: 12 from any source, including microbial sources, can be used in the assays exemplified herein. Desirably such nucleotide sequences and proteins are derived from fungi. More desirably, they are derived from filamentous fungi, particularly Ashbya gossypii. Alternatively, such nucleotide sequences and proteins are derived from non-yeast sources, alternatively from non-Saccharomyces cervisiae sources.

A simple assay can be developed to screen for compounds that affect normal functioning of the fungal-encoded activity. Such compounds are promising in vitro leads that can be tested for in vivo pesticidal, particularly fungicidal, activity. A nucleic acid sequence of the invention according to any one of the sequnces SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11 may be operably linked to a strong inducible promoter, such promoters being known in the art. The vector comprising the selected gene of the invention operably linked to the selected inducible promoter may be transformed into bacteria, such as E. coli. Transformed E. coli harboring and functionally overexpressing expressing a AG001, AG002, AG003, AG004, AG005 or AG006 gene may be grown in a 96-well format for automated high-throughput screening where inducible over expression of the selected gene is lethal or suppresses growth of the host. Compounds that are effective in blocking function of the AG001, AG002, AG003,

AG004, AG005 or AG006 protein results in bacterial growth. This growth is measured by simple turbidometric means.

In another embodiment, an assay for inhibitors of the AG001, AG002, AG003, AG004, AG005 or AG006 activities uses transgenic fungi or fungal cells capable of overexpressing a nucleotide sequence having AG001, AG002, AG003, AG004, AG005 or AG006 activity respectively operably linked to a strong inducible promoter e.g. , wherein the selected gene product is enzymatically active in the transgenic fungi and/or fungal cells and inducible overexpression of the gene inhibits and/ or suppresses growth and/or development of the fungus. The nucleotide sequence is preferably derived from an eukaryote, such as a yeast, but is preferably derived from a fungus and more particularly from a filamentous fungus. In a further preferred embodiment, the nucleic acid sequences set forth in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11 SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11 encode enzymes having AG001, AG002, AG003, AG004,AG005 or AG006 activity respectivelyy, whose amino acid sequence is identical or substantially similar to the amino acid sequence set forth in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10 OR SEQ ID NO: 12. The transgenic fungus or fungal cells are grown in 96-well format microtiter dishes for high-throughput screening. Compounds that are effective in blocking function of the AG001, AG002, AG003, AG004, AG005 or AG006 protein results in fungal growth. This growth is measured by methods known in the art. In a particular embodiment the transgenic fungus is Ashbya gossypii.

Similar assays based on expression of the fungal genes of the invention in yeast, using appropiate expression systems as described above may also be used.

In Vitro Inhibitor Assays: Discovery of Small Molecule Ligand that Interacts with Protein of Unknown Function

Novel technologies are being examined that can detect interactions between a protein and a ligand without knowing the biological function of the protein. A short description of three methods is presented, including fluorescence correlation spectroscopy, surface-enhanced laser desorption/ionization, and biacore technologies. Many more of these methods are currently being discovered, and some may be amenable to automated, large scale screening in light of this disclosure.

Fluorescence Correlation Spectroscopy (FCS) theory was developed in 1972 but it is only in recent years that the technology to perform FCS became available (Madge et al. (1972) Phys. Rev. Lett., 29: 705-708; Maiti et al. (1997) Proc. Natl. Acad. Sci. USA, 94: 11753-11757). FCS measures the average diffusion rate of a fluorescent molecule within a small sample volume. The sample size can be as low as 103 fluorescent molecules and the sample volume as low as a the cytoplasm of a single bacterium. The diffusion rate is a function of the mass of the molecule and decreases as the mass increases. FCS can therefore be applied to protein-ligand interaction analysis by measuring the change in mass and therefore in diffusion rate of a molecule upon binding.

Surface-Enhanced Laser Desorption/Ionization (SELDI) was invented by Hutchens and Yip during the late 1980's (Hutchens and Yip (1993) Rapid Commun. Mass Spectrom. 7: 576-580). When coupled to a time-of-flight mass spectrometer (TOF), SELDI provides a mean to rapidly analyze molecules retained on a chip. It can be applied to ligand-protein interaction analysis by covalently binding the target protein on the chip and analyze by MS the small molecules retained by this protein (Worrall et al. (1998) Anal. Biochem. 70: 750-756).

Biacore relies on changes in the refractive index at the surface layer upon binding of a ligand to a protein immobilized on the layer. In this system, a collection of small ligands is injected sequentially in a 2-5 ul cell with the immobilized protein. Binding is detected by surface plasmon resonance (SPR) by recording laser light refracting from the surface. In general, the refractive index change for a given change of mass concentration at the surface layer, is practically the same for all proteins and peptides, allowing a single method to be applicable for any protein (Liedberg et al. (1983) Sensors Actuators 4: 299-304; Malmquist (1993) Nature, 361: 186-187).

IV. In Vivo Inhibitor Assay

In one embodiment, a suspected pesticide, particularly fungicide, for example identified by in vitro screening, is applied to fungi at various concentrations. After application of the suspected fungicide, its effect on the fungus, for example inhibition or suppression of growth and development is recorded.

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLES

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, et al., Molecular Cloning, eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) and by T.J. Silhavy, M.L. Berman, and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F.M. et al., Current Protocols in Molecular Biology, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987),

Construction and characterization of a Genomic Library of A. gossypii (strain ATCC10895), identification of ORF and promoters is described in U.S. Patent Application Ser. No.: 08/998,416 which is hereby incorporated by reference in its entirety.

Example 1: Identification of Antifungal Drug Targets Represented in the Sequence Listing

Gene disruptions of *Ashbya gossypii* genes are generated by a method using short flanking homology regions to produce gene targeting events. The short flanking homology regions are included within polymerase chain reaction primers of 65 nucleotide overall sequence length. Each of these 65-mers contains approximately 45 nucleotides homology to the target gene locus the target gene locus being identified as described in U.S. Patent Application Ser. No. 08/998,416 incorporated above by reference, and 20 nucleotides homology (invariant) to a geneticin resistance gene module(also described in U.S. Patent Application Ser. No. 08/998,416 previously incorporated by reference) , with one primer (designated S1) anchored to the 5' end of the geneticin resistance module (using the invariant sequence 5'-GCTAGGGATAACAGGGTAAT-3') (SEQ ID NO:13)and the other primer of the pair (designated S2) anchored to the 3' end of the geneticin resistance module (using the invariant sequence 5'-AGGCATGCAAGCTTAGATCT-3') (SEQ ID NO:14). The PCR product resulting from the amplification of the geneticin resistance module with such

an S1/S2 primer pair thus consists of the module flanked by short flanking homology regions of ca. 45 nucleotides specific to the chosen gene disruption site.

Once an S1/S2 primer pair is designed for a particular gene target, approximately 10 ug of the desired geneticin resistance module is obtained by linearizing a vector containing the geneticin resistance gene positioned behind the an appropriate fungal promoter (for example, the Saccharomyces cerevisiae TEF1 promoter) and subjecting the linearized template to approximately 35 rounds of a PCR reaction consisting of the following steps: Step 1: Denaturation at 96 C for 30 seconds; Step 2: Primer annealing at 50 C for 30 seconds; Step 3: Elongation reaction at 72 C for 2.5 minutes. Following the 35th round of this protocol, a final elongation period of 5 minutes at 72 C is carried out.

Transformation of the PCR product resulting from amplification with the S1/S2 primer pair is done by electroporation as follows: 1) Inoculate 100 ml of AFM media (1% casein peptone, 2% glucose, 1% yeast extract, 0.1% myo-inositol) with an Ashbya spore suspension of approximately 10^7 spores. 2) Incubate at 30 C for a maximum of 18 hors at a shaker speed of 200 rpm. 3) Collect the resultant fungal mycelia by filtration and wash once with sterile water. 4) Resuspend 1 gram of mycelia (wet weight) in 40 ml of 50 mM potassium phosphate buffer, pH 7.5 containing 25mM DTT and incubate at 30 C for 30 minutes with gentle shaking. 5) Collect the mycelia by filtration and wash once with 50 ml of cold STM buffer (275 mM sucrose, 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$). 6) Resuspend the mycelia to a dense mixture in STM buffer. 7) Mix approximately 150 ul of the mycelial mixture with 10 ug of PCR product (in a maximum volume of 50 ul) in an Eppendorf tube and transfer the mixture to an electroporation cuvette with a 4mM gap distance. 8) Apply an electric field pulse of 1.5 kV, 100 ohms, 25 uF which will result in a pulse length of approximately 2.3 milliseconds. Add 1 ml of AFM media to the cuvette and spread equal amounts onto 3 pre-dried AFM agar plates. 9) Incubate plates for a minimum of 4 hours at 30 C. 10) Overlay the plates with 8 ml of a 0.5%agarose toplayer containing Geneticin/G418 at a final concentration of 200 ug/ml. 11) Incubate at 30 C for approximately 3 days to allow sufficient growth of geneticin resistant transformants.

Verification of the desired transformation event resulting in homologous integration of the geneticin resistance module in the target of interest is achieved by PCR using verification primers designated G1 (positioned upstream of the S1 region) and G4 (positioned downstream of the S2 region) and template DNA purified from putative *Ashbya* transformants. Additional verification primers designated G2 (5'-GTTTAGTCTGACCATCTCATCTG-3') (SEQ ID NO15)and G3 (5'-

TCGCAGACCGATACCAGGATC-3') (SEQ ID NO:16) are derived from the open reading frame of the selectable geneticin resistance gene such that the detection of a G1/G2 PCR product and or a G3/G4 PCR product of a predictable size serves to verify the desired gene disruption event. Also, verification of the desired gene disruption can be determined by standard DNA hybridization experiments.

Determination of whether a gene is essential to growth of *Ashbya* can be achieved by the following analysis. The transformation of DNA fragments described above utilizes multinucleate *Ashbya* mycelia as recipients. Therefore a primary transformant able to grow on geneticin containing media originates as a mycelium containing cells at least one of which has at least one transformed nucleus, but usually containing non-transformed nuclei as well. Thus, if an essential gene is disrupted in the transformed nucleus, the essential gene product can, in many instances, still be supplied by the non-transformed nuclei within the same cell. Such primary transformants usually exhibit normal growth and sporulation, and spores are collected from primary transformants allowed to grow at 30 C for at least 5 days. Since spores are uninucleate, however, transformants which have an essential gene disrupted in nuclei containing the geneticin resistance cartridge will fail to yield spores which grow normally, if at all, on geneticin-containing media.

S1 and S2 primer pairs usable to generate disruptions of the indicated genes are as follows:

AG001: S1: 5'-AGGACCACTAGCTCGTTGCGCTGCAATATAATAATAAGAACGAGA GCTAGGGATAACAGGGTAAT-3' (SEQ ID NO:17)

S2: 5'-AAGTATTCAATCAACTATGTGAGTAGTTTCTTGTAGGCAGTCTCC AGGCATGCAAGCTTAGATCT-3'(SEQ ID NO:18)

AG002: S1: 5'-CTGGCATCAGAGGAAGCTCCCACCACCAAGCTCTACAAACACAAG GCTAGGGATAACAGGGTAAT-3'(SEQ ID NO:19)

S2: 5'-ATTATATTAGTATAGTCTAAAGTTGCAGGCAGTGGGTATTAAAGT AGGCATGCAAGCTTAGATCT-3'(SEQ ID NO:20)

AG003: S1: 5'-ACTTGCGTACTCTTTCGCGTGCTCGTCAGCCACCGAACAACGCAG GCTAGGGATAACAGGGTAAT-3'(SEQ ID NO:21)

S2: 5'-TTAAAGAATGATAAAGAACCAAAAACACCACGAGCTTGCATAACA AGGCATGCAAGCTTAGATCT-3'(SEQ ID NO:22)

AG004: S1: 5'-GTGCGTGTCAGCGAGCATCTAATCAAGCTGCAAGGCGCCGGAAAT GCTAGGGATAACAGGGTAAT-3'(SEQ ID NO:23)

S2: 5'-TTATCACATATTTCTAAGTTAATAGATATTTTTACTTAGTATGAA AGGCATGCAAGCTTAGATCT-3'(SEQ ID NO:24)

AG006: S1: 5'-GAGAGAGACGCTACGGTACTACGAATTTCTCTGTAGAGTTGGAGA GCTAGGGATAACAGGGTAAT-3'(SEQ ID NO:25)

S2: 5'-TACTATTGAGAATGTTCGCGACTGCATGTAAAGTCTCAAAAACTT AGGCATGCAAGCTTAGATCT-3'(SEQ ID NO:26)

AG005: S1: 5'-AAATATAATAAAAATTGACAACTGGCTAGAAGTGATACCGCAGTT GCTAGGGATAACAGGGTAAT-3'(SEQ ID NO:27)

S2: 5'-CCTCTTATAGTTCATGACCCATTCATATGCGTCATTCAGGTCTCT AGGCATGCAAGCTTAGATCT-3'(SEQ ID NO:28)

The above disclosed embodiments are illustrative. This disclosure of the invention will place one skilled in the art in possession of many variations of the invention. All such obvious and foreseeable variations are intended to be encompassed by the appended claims.

What is claimed is:

1. A nucleotide sequence substantially similar to any one of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11.

2. The nucleotide sequence of claim 1, wherein the nucleotide sequence is SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11.

3. The nucleotide sequence of claim 1 or 2, wherein the nucleotide sequence is a fungal nucleotide sequence.

4. The nucleotide sequence of claim 3, wherein the fungus is Ashbya gossypii.

5. The nucleotide sequence of claim 1, wherein the nucleotide sequence encodes an amino acid sequence substantially similar to any one of SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, SEQ ID NO:10 or SEQ ID NO:12 respectively.

6. A nucleotide sequence encoding an amino acid sequence according to any one of SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, SEQ ID NO:10 or SEQ ID NO:12.

7. A nucleotide sequence having a 20 base pair nucleotide portion identical in sequence to a 20 consecutive base pair portion of a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11.

8. An amino acid sequence comprising an amino acid sequence encoded by a nucleic acid sequence substantially similar to any one of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 OR SEQ ID NO: 11.

9. An amino acid sequence substantially similar to any one of SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, SEQ ID NO:10 or SEQ ID NO:12.

10. A chimeric gene comprising a promoter operably linked to a nucleotide sequence according to claim 1 or 2.

11. The chimeric gene of claim 10'8 wherein the promoter is an inducible promoter.

12. A recombinant vector comprising a chimeric gene according to claim 11 wherein said vector is capable of being stably transformed into a host cell.

13. A host cell comprising the vector according to claim 12, wherein the nucleotide sequence is expressible in the host cell.

14. The host cell according to claim 13, wherein the host cell is an Ashbya gossipii cell.

15. A process for identifying compounds having fungicidal activity comprising the steps of:

a) combining a protein according to claim 16 and a compound to be tested for the ability to bind to the protein, under conditions having conducive binding,

b) selecting a compound identified by step a) that is capable of binding the protein;

c) applying the identified compound from step b) to a fungus to test for fungicidal activity; and

d) selecting compounds having fungicidal activity.

16. A compound having fungicidal activity identifiable according to claim 15.

17. A process for identifying an inhibitor of a protein activity having an amino acid sequence according to claim 9 comprising:

a) introducing SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 or SEQ ID NO: 11 or nucleotide sequences substantially similar thereto into a host, such that the sequence is functionally expressible;

b) combining the host cell of step a) with a compound to be tested for ability to inhibit the protein activity;

c) over expressing the nucleotide sequence of step a);

c) measuring the host cell growth in stepc); and

d) selecting the compound that inhibits or suppresses host cell's normal growth or development in step c).

18. A compound having fungicidal activity identifiable according to the process of claim 170.

19. A method of suppressing growth of a fungus comprsing applying to the fungus a compound that inhibits the activity of a protein comprising the amino acid sequence according to claim 9 in an amount sufficient to suppress the growth of the fungus.

20. The method of claim 19 wherein the compound is selected from a group consisting of the compounds of claims 16 and claim 18.

SEQUENCE LISTING

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Wendland, Juergen
Philippsen, Peter

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Ser Pro Asn Ser Lys Asn Asn Met Ser Arg His Ser His Ser Asn Gly
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Gln Gln Gln Pro Gln Thr Pro Met Gln Leu Gln Val Pro Thr Gly Gln
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115 120 125

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225 230 235 240

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Lys Ile Asn Leu Thr Phe Asn Arg Val Trp Ser Pro Gln Ala Asp Tyr
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820 825 830

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850 855 860

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865 870 875 880

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885 890 895

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Tyr Asp Glu Leu Lys Phe Pro Val Trp Asp Leu Lys Val Thr Ser Val
1075 1080 1085

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1475 1480 1485

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1585 1590 1595 1600

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1635 1640 1645

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1650 1655 1660

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1665 1670 1675 1680

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1685 1690 1695

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1700 1705 1710

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1730 1735 1740

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1745 1750 1755 1760

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1765 1770 1775

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1780 1785 1790

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1795 1800 1805

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1810 1815 1820

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1825 1830 1835 1840

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Met His Glu Trp Met Lys Ala Ile Thr Leu Ser Lys Arg Tyr Ser Phe
1845 1850 1855

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His Ser Lys Arg Phe Lys Gly Lys Thr Ser Asn Lys Ile Phe Gly Val
1860 1865 1870

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Pro Val Glu Asp Val Cys Glu Arg Glu Gly Ala Leu Ile Pro Asn Ile
1875 1880 1885

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Ile Val Lys Leu Leu Asp Glu Ile Glu Leu Arg Gly Leu Asp Glu Val
1890 1895 1900

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Gly Leu Tyr Arg Val Pro Gly Ser Val Gly Ser Ile Asn Ala Leu Lys
1905 1910 1915 1920

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Asn Ala Phe Asp Asp Glu Gly Ala Val His Asn Thr Phe Thr Leu Glu
1925 1930 1935

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Asp Asp Arg Trp Phe Glu Ile Asn Thr Ile Ala Gly Cys Phe Lys Leu
1940 1945 1950

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Tyr Leu Arg Glu Leu Pro Glu Ser Leu Phe Thr Asn Glu Lys Val Asp
1955 1960 1965

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965 970 975

aac ata ccc gtg aat ata cta cat ccg ttt atc act gac ttt ggc tgt 2976
Asn Ile Pro Val Asn Ile Leu His Pro Phe Ile Thr Asp Phe Gly Cys
980 985 990

ata ttc caa gat aag gcg ccg atg gag aac gga cca ccg gtc aac ata 3024
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Pro Thr Tyr Glu Glu Leu Val Glu Glu Asn Val Gln Leu Arg Arg Glu
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Leu Gln Gly Gln Arg Glu Glu Ile Glu His Leu Arg Lys Thr Ile Ser
65 70 75 80

Leu Leu Ala Ser Gly Arg Ser Gly Ala Thr Val Val Glu Gln Gln Val
85 90 95

Arg Pro Glu Pro Ser Pro Ser Val Arg Glu Leu Ala Leu Pro Pro Arg
100 105 110

Ser Ala Asp Arg Arg Lys Asn Thr Lys Asn Leu Ser Leu Ala Pro Val
115 120 125

Gly His Glu Val Pro Ser Thr Asp Arg Leu Arg Val Ser Pro Gln Glu
130 135 140

Ala Thr Ser Gly Ala Gln Gln Val Pro Leu Leu Thr Ser Ser Lys Ser
145 150 155 160

Ala Glu Ile Leu Val Ser Lys Ser Pro Asp Glu Asp Arg His Leu Met
165 170 175

Ser Pro Arg Lys Thr Ile Ser Arg Ser Ser Ser Ser Tyr Ser Asn Thr
180 185 190

Leu Gly Ser Pro Ala Thr Ser Val Leu Tyr Lys Asn Ser Arg Ile Ser

195 200 205

Ile Thr Ser Pro Cys Lys Ser Asn Ser Thr Ser Lys Ala Ala Ser Val
210 215 220

Leu Ser Leu Pro Glu Asn Asn Thr Ser Thr Glu Asn Ala Pro His Ser
225 230 235 240

Pro His Arg Ile Asp Asn Glu Leu Asp Leu Leu Thr Val Glu Pro Gln
245 250 255

Asp Gly Ser Arg Tyr Asp Thr Glu Arg Ala Gly Gly Pro Gly Pro Leu
260 265 270

Ser Pro Glu Ser Ile Val Tyr Ser Asp Ser Asp Leu Gln Glu His Gln
275 280 285

Pro Ser Asp Leu Ser Ser Thr Thr Arg Thr Asp Leu Gly Lys Phe Arg
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Asp Met Val Asp Thr Thr Phe Asn Ala Glu Asp Asn Pro Thr Gly Ser
305 310 315 320

Arg Asp Lys Glu Thr Gly Thr Glu Met Glu Ile Ala Thr Leu Gln Asn
325 330 335

Thr Pro Ser Arg Gln His Glu Ser Ser Leu Val Thr Ser Pro Gln Ala
340 345 350

Ser Arg Ser Ser Ile Thr Thr Pro Val Val Asp Pro Thr Asn Thr Ser
355 360 365

Glu Pro Ser Ser Leu Ser Ala Ala Lys Phe Gly Ser Met Ser Thr Ala

370 375 380

Thr Ser Ser Asn Lys Arg Ser Lys Gly Met Gly Thr Pro Ser Val Glu
385 390 395 400

His Ser Ala Lys Ser Tyr Ser Gln His Ser Gly Ser Pro His Ser Asn
405 410 415

Ser His Gln Ser Lys Lys Ala Asp Ile Pro Leu Phe Val Gln Pro Glu
420 425 430

Glu Leu Gly Thr Ile Arg Ile Glu Val Ile Ser Thr Leu Tyr His Glu
435 440 445

Pro Gly Asn Ala Ala Ser Ile Leu Phe Ser Val Val Asp Lys Lys Ser
450 455 460

Ser Lys Glu Met Phe Lys Phe Ala Lys Thr Phe Thr Arg Ile Ala Glu
465 470 475 480

Phe Asp Thr Phe Ile Arg Asn Asn Met Glu Ser Leu Ala Val Pro Pro
485 490 495

Leu Pro Asp Lys His Met Phe Ala Ser Asn Val Pro Val Lys Val Asp
500 505 510

Ser Arg Arg Glu Lys Leu Asn Asp Tyr Phe Ala Ser Leu Leu Tyr Leu
515 520 525

Ser Pro Leu Pro Phe Asn Pro Ala Leu Lys Leu Ala Gln Phe Ile Ser
530 535 540

Thr Asp Pro Val Met Asn Pro Ile Thr Gly Glu Phe Ala Lys Glu Gly

545 550 555 560

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565 570 575

Arg Ile Arg Tyr Cys Thr Val Glu Gly Ser Ile Met His Leu His Asp
580 585 590

His Met Ile Asp Thr Asp Thr Ile Lys Leu Thr His Ser Thr Ile Glu
595 600 605

Leu Gln Ala Asn Leu Pro Asp Asp Lys Tyr Gly Thr Lys Asn Gly Phe
610 615 620

Ile Leu Asn Glu His Lys Lys Ser Gly Leu Ser Ser Ser Thr Lys Tyr
625 630 635 640

Tyr Phe Cys Ala Glu Thr Pro Lys Glu Arg Glu Gln Trp Ile Ser Val
645 650 655

Leu Thr Thr Leu Cys Asp Gly Pro Gly Gly Thr Ala Ala Ile Pro Ser
660 665 670

Ile Asn Ser Lys Ser Glu Ala Ser Ser Leu Phe Glu Gln Thr Ser Ile
675 680 685

Ser Asp Ser Ser Tyr Leu Gly Pro Ile Ala Asn Leu Glu Ala Met Asp
690 695 700

Ala Thr Ser Pro Thr Arg Pro Asn Asp Pro Asn Pro Val Ser Leu Thr
705 710 715 720

Ser Glu Glu Glu Lys Glu Val Lys Arg Arg Arg Met Lys Ser Phe Phe

725 730 735

Pro Phe Lys Lys Leu Ala Thr Thr Pro Thr Pro Tyr Ala Ala Gly Asn
740 745 750

Asp Asn Ala Ser Ile Phe Ser Gln Asp Asp Asp Ser Pro Val Asn Ala
755 760 765

Thr Asn Glu Ser Gly Ile Ser Arg Ser Leu Gln Ser Met Asn Leu Gln
770 775 780

Ala Gln Tyr Asn Ala Val Phe Gly Ala Asp Leu Arg Ser Cys Leu Gln
785 790 795 800

Leu Ser Ser His Pro Tyr Gln Gly Lys Tyr Glu Ile Pro Ser Val Val
805 810 815

Phe Arg Thr Leu Glu Phe Leu Tyr Lys Asn Arg Gly Ile Gln Glu Glu
820 825 830

Gly Ile Phe Arg Leu Ser Gly Ser Ser Ser Leu Ile Lys Ser Leu Gln
835 840 845

Glu Gln Phe Asp Lys Glu Tyr Asp Val Asp Leu Cys Asn Tyr Asn Asp
850 855 860

Lys Val Ser Val Thr Pro Gly Asn Glu Asn Gln Gly Gly Leu Tyr Val
865 870 875 880

Asp Val Asn Thr Val Ser Gly Leu Leu Lys Leu Tyr Leu Arg Lys Leu
885 890 895

Pro His Met Ile Phe Gly Asp Ala Ala Tyr Met Asp Phe Lys Arg Ile

900 905 910

Val Glu Arg Asn Gly Asp Asp Ser Lys Leu Ile Ala Leu Glu Phe Arg
915 920 925

Ala Leu Val Asn Ser Gly Arg Ile Ala Lys Glu Tyr Val Ala Leu Met
930 935 940

Tyr Ala Leu Phe Glu Leu Leu Val Lys Ile Thr Glu Asn Ser Lys Tyr
945 950 955 960

Asn Lys Met Asn Leu Arg Asn Leu Cys Ile Val Phe Ser Pro Thr Leu
965 970 975

Asn Ile Pro Val Asn Ile Leu His Pro Phe Ile Thr Asp Phe Gly Cys
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995 1000 1005

His Ile Pro Gln Ile
1010

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<212> DNA
<213> Ashbya gossypii

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gaa gtg ata ccg cag ttg ata tcc cga att cac cag cct aac caa acc 96
Glu Val Ile Pro Gln Leu Ile Ser Arg Ile His Gln Pro Asn Gln Thr
20 25 30

gtg agt aga aca tta tta tct ctc tta tct gac ctc ggc aag gct cat 144
Val Ser Arg Thr Leu Leu Ser Leu Leu Ser Asp Leu Gly Lys Ala His
35 40 45

cct cag gct ctc gtc ttc cct cta aca gtt gct ata aaa tct gaa tct 192
Pro Gln Ala Leu Val Phe Pro Leu Thr Val Ala Ile Lys Ser Glu Ser
50 55 60

gta tct agg cag aga gct gct ttg tct att atg gag aag atg cgt atg 240
Val Ser Arg Gln Arg Ala Ala Leu Ser Ile Met Glu Lys Met Arg Met
65 70 75 80

cat agt tct aat ctg gtt gaa cag gca gaa ctg gtt agc aat gag ctc 288
His Ser Ser Asn Leu Val Glu Gln Ala Glu Leu Val Ser Asn Glu Leu
85 90 95

att cgt att gct gtg ctg tgg cat gag cta tgg tat gaa ggt ctg gag 336
Ile Arg Ile Ala Val Leu Trp His Glu Leu Trp Tyr Glu Gly Leu Glu
100 105 110

gac gcg agt aga cag ttt ctc gga gag cat aat acg gaa aag atg ttc 384
Asp Ala Ser Arg Gln Phe Leu Gly Glu His Asn Thr Glu Lys Met Phe
115 120 125

gct act ttg gaa cca ctg cat gaa atg ttg aag agg gga cct gag act 432
Ala Thr Leu Glu Pro Leu His Glu Met Leu Lys Arg Gly Pro Glu Thr
130 135 140

cta cgg gag ata tca ttc cag aat tca ttt ggt aga gac ctg aat gac 480
Leu Arg Glu Ile Ser Phe Gln Asn Ser Phe Gly Arg Asp Leu Asn Asp
145 150 155 160

gca tat gaa tgg gtc atg aac tat aag agg aca cag gat atc agt aat 528
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tt 530

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<211> 176
<212> PRT
<213> Ashbya gossypii

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20 25 30

Val Ser Arg Thr Leu Leu Ser Leu Leu Ser Asp Leu Gly Lys Ala His
35 40 45

Pro Gln Ala Leu Val Phe Pro Leu Thr Val Ala Ile Lys Ser Glu Ser
50 55 60

Val Ser Arg Gln Arg Ala Ala Leu Ser Ile Met Glu Lys Met Arg Met
65 70 75 80

His Ser Ser Asn Leu Val Glu Gln Ala Glu Leu Val Ser Asn Glu Leu
85 90 95

Ile Arg Ile Ala Val Leu Trp His Glu Leu Trp Tyr Glu Gly Leu Glu
100 105 110

Asp Ala Ser Arg Gln Phe Leu Gly Glu His Asn Thr Glu Lys Met Phe
115 120 125

Ala Thr Leu Glu Pro Leu His Glu Met Leu Lys Arg Gly Pro Glu Thr
130 135 140

Leu Arg Glu Ile Ser Phe Gln Asn Ser Phe Gly Arg Asp Leu Asn Asp
145 150 155 160

Ala Tyr Glu Trp Val Met Asn Tyr Lys Arg Thr Gln Asp Ile Ser Asn
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<212> DNA
<213> Ashbya gossypii

<220>
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<222> (1)..(402)

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1 5 10 15

ctc tgt aga gtt gga gag gca aaa cgt tgg att gag gat gtg atc ggc 96
Leu Cys Arg Val Gly Glu Ala Lys Arg Trp Ile Glu Asp Val Ile Gly
20 25 30

gag acg ata cct gga gaa ctc gag ttg gca gct ggt aat tca atg cgc 144
Glu Thr Ile Pro Gly Glu Leu Glu Leu Ala Ala Gly Asn Ser Met Arg
35 40 45

gac ggc tat ttt ttg gcg aag gtc act caa acg att aaa cct gat ctt 192
Asp Gly Tyr Phe Leu Ala Lys Val Thr Gln Thr Ile Lys Pro Asp Leu
50 55 60

gca cct aca att gta cct cct ggt cgg ttg cag ttc aag cat aca cag 240
Ala Pro Thr Ile Val Pro Pro Gly Arg Leu Gln Phe Lys His Thr Gln
65 70 75 80

aat att aat gct ttt ttt tcg ctg atg gat ttg gta ggc gta ccg gac 288
Asn Ile Asn Ala Phe Phe Ser Leu Met Asp Leu Val Gly Val Pro Asp
85 90 95

cta ttt cga ttt gaa ctg acc gac cta tac gag aag aaa gac gtt cca 336
Leu Phe Arg Phe Glu Leu Thr Asp Leu Tyr Glu Lys Lys Asp Val Pro
100 105 110

aaa gtt ttt gag act tta cat gca gtc gcg aac att ctc aat agt agg 384
Lys Val Phe Glu Thr Leu His Ala Val Ala Asn Ile Leu Asn Ser Arg
115 120 125

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Phe Pro Gly Glu Ile Pro

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20 25 30

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35 40 45

Asp Gly Tyr Phe Leu Ala Lys Val Thr Gln Thr Ile Lys Pro Asp Leu
50 55 60

Ala Pro Thr Ile Val Pro Pro Gly Arg Leu Gln Phe Lys His Thr Gln
65 70 75 80

Asn Ile Asn Ala Phe Phe Ser Leu Met Asp Leu Val Gly Val Pro Asp
85 90 95

Leu Phe Arg Phe Glu Leu Thr Asp Leu Tyr Glu Lys Lys Asp Val Pro
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<223> Description of Artificial Sequence:Primer

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<223> Description of Artificial Sequence:Primer

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<211> 21
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<210> 18
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gatct 65

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gtaat 65

<210> 20
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<210> 21
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gtaat 65

<210> 22
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<213> Artificial Sequence

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gatct 65

<210> 25

<211> 65
<212> DNA
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<210> 26
<211> 65
<212> DNA
<213> Artificial Sequence

<220>
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gatct 65

<210> 27
<211> 65
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<223> Description of Artificial Sequence:Primer

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gtaat 65

<210> 28
<211> 65
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Primer

<400> 28
cctcttatag ttcatgaccc attcatatgc gtcattcagg tctctaggca tgcaagctta 60

gatct 65

INTERNATIONAL SEARCH REPORT

Interna[redacted]l Application No
PCT/[redacted]99/07501

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C07K14/37 G01N33/53 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 866 129 A (CIBA GEIGY AG) 23 September 1998 (1998-09-23) see whole document, particularly seq.ID.80 ---	1,3-5,7
Y	MADAULE P ET AL: "CHARACTERIZATION OF TWO MEMBERS OF THE RHO GENE FAMILY FROM THE YEAST SACCHAROMYCES CEREVISIAE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 84, page 779-783 XP002038042 ISSN: 0027-8424 the whole document --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 January 2000	01.03.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ALTMANN-JÖHL, R. ET AL.: "AgTHR4, a new selection marker for transformation of the filamentous fungus Ashbya gossypii, maps in a four-gene cluster that is conserved between A. gossypii and Saccharomyces cerevisiae." MOLECULAR AND GENERAL GENETICS, vol. 250, 1996, pages 69-80, XP002127169 the whole document, particularly the abstract. ---	1-9
Y	MATSUI, Y. ET AL.: "Isolation and characterization of two novel ras superfamily genes in Saccharomyces cerevisiae." GENE, vol. 114, 1992, pages 43-9, XP002127170 the whole document ---	1-9
A	DATABASE NCBI [Online] Acc.no. U09322, 25 May 1994 (1994-05-25) MESSNER, R. ET AL.: "Ashbya gossypii strain HA88 internal transcribed spacer 1 (ITS1) and 2 (ITS2) and 5.8S rRNA gene, complete sequence." XP002127172 the whole document ---	
A	STEINER, S. ET AL.: "Sequence and promoter analysis of the highly expressed TEF gene of the filamentous fungus Ashbya gossypii." MOLECULAR AND GENERAL GENTICS, vol. 242, 1994, pages 263-71, XP002127171 the whole document ---	
A	WO 98 29538 A (ALTMANN JOEHL REGULA ;PHILIPPSEN PETER (CH); ALTHOEFER HENNING (DE) 9 July 1998 (1998-07-09) the whole document -----	

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/07501

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 16,18-20
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

A meaningful search of claims 16 and 18 was not possible due to a lack of characterization of the claimed fungicidal compounds and inhibitors. Claims 19 and 20, relating directly and exclusively to these compounds and their use, could not be searched either.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Claims 1-20 (all partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 99/07501

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 16,18-20

A meaningful search of claims 16 and 18 was not possible due to a lack of characterization of the claimed fungicidal compounds and inhibitors. Claims 19 and 20, relating directly and exclusively to these compounds and their use, could not be searched either.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/ EP 99/07501

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-20, all partially

The GTP-binding protein-like genes from Ashbya gossypii with nucleic acid sequences represented in seq.ID's 1 and 3, encoding the respective protein seq.ID's 2 and 4, nucleic acids or proteins substatially similar to said nucleic acids and proteins, nucleic acids comprising at least 20 consequtive nucleotides from said sequences, chimeric genes and vectors comprising said nucleic acids, host cell transformed with said vector, process for producing said protein using said host cell, process for identifying inhibitors of said protein's activity, inhibotirs identified by said process, and method of suppressing growth of a fungus by using said inhibitors.

2. Claims: 1-20, all partially

The GTPase activating protein-like genes from Ashbya gossypii with nucleic acid sequences represented in seq.ID's 5 and 7, encoding the respective protein seq.ID's 6 and 8, nucleic acids or proteins substatially similar to said nucleic acids and proteins, nucleic acids comprising at least 20 consequtive nucleotides from said sequences, chimeric genes and vectors comprising said nucleic acids, host cell transformed with said vector, process for producing said protein using said host cell, process for identifying inhibitors of said protein's activity, inhibotirs identified by said process, and method of suppressing growth of a fungus by using said inhibitors.

3. Claims: 1-20, all partially

The phosphatidylinositol-4-kinase-like gene from Ashbya gossypii with nucleic acid sequences represented in seq.ID 9, encoding the respective protein seq.ID 10, nucleic acids or proteins substatially similar to said nucleic acids and proteins, nucleic acids comprising at least 20 consequtive nucleotides from said sequences, chimeric genes and vectors comprising said nucleic acids, host cell transformed with said vector, process for producing said protein using said host cell, process for identifying inhibitors of said protein's activity, inhibotirs identified by said process, and method of suppressing growth of a fungus by using said inhibitors.

4. Claims: 1-20, all partially

The cytokinesis-like gene from Ashbya gossypii with nucleic acid sequences represented in seq.ID 11, encoding the

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

respective protein seq.ID 12, nucleic acids or proteins substatially similar to said nucleic acids and proteins, nucleic acids comprising at least 20 consequtive nucleotides from said sequences, chimeric genes and vectors comprising said nucleic acids, host cell transformed with said vector, process for producing said protein using said host cell, process for identifying inhibitors of said protein's activity, inhibotirs identified by said process, and method of suppressing growth of a fungus by using said inhibitors.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna[redacted]l Application No
PCT/[redacted]9/07501

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0866129 A	23-09-1998	AU 5764398 A	31-07-1998
		AU 6291698 A	31-07-1998
		WO 9829538 A	09-07-1998
		WO 9829539 A	09-07-1998
		EP 0951538 A	27-10-1999
		EP 0953044 A	03-11-1999
		JP 11225770 A	24-08-1999
WO 9829538 A	09-07-1998	AU 5764398 A	31-07-1998
		AU 6291698 A	31-07-1998
		WO 9829539 A	09-07-1998
		EP 0866129 A	23-09-1998
		EP 0951538 A	27-10-1999
		EP 0953044 A	03-11-1999
		JP 11225770 A	24-08-1999